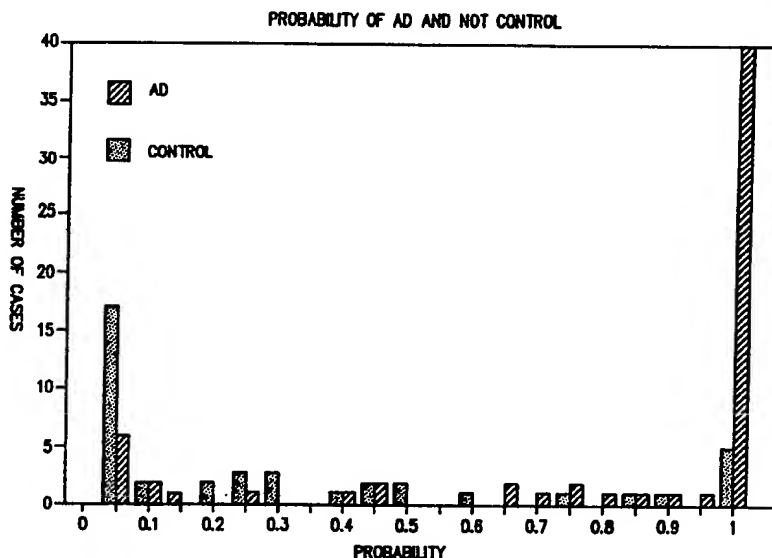




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(54) Title: METHOD OF DIAGNOSING OR CATEGORIZING DISORDERS FROM BIOCHEMICAL PROFILES**(57) Abstract**

A method for diagnosing disorders in living organisms, in which fluid samples from normal and afflicted (abnormal) individuals are analyzed to generate patterns representative of molecular constituents of said samples. A data base of frequency distribution patterns of constituents of samples from organisms having known categories of disorders and controls is created, and the unknown sample analysis is compared for conformity to said frequency distribution patterns. The invention has particular applicability to diagnosing degenerative diseases such as Alzheimer's Disease, Parkinson's Disease, Huntington's Disease, schizophrenia, Progressive Supranuclear Palsy, amyotrophic lateral sclerosis and senile dementia, which are given as exemplary. The invention also may be advantageously employed to diagnose diseases such as tumors, carcinomas, cardiovascular abnormalities and other disorders, or for selection of the therapy based on categories of known vs. unsuccessful outcomes. Moreover, both treatment protocols and new pharmaceuticals may be evaluated.

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1 METHOD OF DIAGNOSING OR CATEGORIZING
2 DISORDERS FROM BIOCHEMICAL PROFILES

3 This invention relates to analytical and mathematical
4 methods for diagnosing or categorizing disorders. The
5 invention has particular utility for diagnosing or
6 categorizing disorders in living animals from analysis
7 profiles of biologically active materials such as
8 neurotransmitters and other neurochemical substances in
9 brain tissue, cerebrospinal fluid, plasma, serum, saliva,
10 nasal mucosa, urine and the like, such as catecholamines,
11 their precursors, cofactors and their metabolites. The
12 invention is uniquely capable of differentiating a large
13 number of compounds of biological, diagnostic and/or
14 pharmaceutical significance and of using such differential
15 for diagnosing disorders and will be described in
16 connection with such utility although other uses are
17 contemplated.

18 There is an extensive body of literature relating
19 abnormalities in neurotransmitters, precursors, and
20 metabolites to degenerative, neuropsychiatric and
21 behavioral disorders, hypertension and certain carcinomas.
22 See, for example, Schildkraut et al in The Brain,
23 Biochemistry and Behavior, Proceedings of the Sixth,
24 Arnold O. Beckman Conference in Clinical Chemistry, pages
25 47-68. Although the potential role of these compounds in
26 a number of significant disorders has been established,
27 their routine analysis has not yet achieved widespread
28 clinical use. Two problems in the clinical utility of
29 neurotransmitter measurements are related to the economic
30 and technical limitations of current technology. First,
31 there is felt to be a high degree of interlaboratory and
32 intersample uncertainty in quantitative values. Second, it
33 has been difficult to measure enough of the known
34 metabolically related compounds of a particular
35 neurotransmitter to fully describe its biochemical
36 significance in an individual sample, or to detect,

1 identify and measure unusual neurotransmitters - an
2 important aspect of basic research in various disease
3 states that is presently very expensive and specialized.

4 While a number of interlaboratory technique
5 intercomparisons for a variety of neurotransmitters have
6 been carried out, there has been no comprehensive study
7 within and among different techniques and laboratories for
8 neurotransmitters in typical samples of interest. In the
9 absence of such studies, given the complexity of the
10 analytical problem and the historically wide variation
11 whenever an analyte has been subjected to rigorous
12 interlaboratory testing, the current values for normal and
13 abnormal neurotransmitter levels must be taken with
14 unspecified and probably wide limits of confidence.

15 Although the analysis of single neurotransmitters or
16 metabolites from a complex biochemical pathway has been
17 shown to correlate with a number of disorders utilizing
18 statistical analysis over a large number of samples, the
19 analytical level of a single neurotransmitter in an
20 individual sample, with a few exceptions, has had
21 relatively low clinical diagnostic utility. Essentially
22 the state of the field of biochemical correlates of
23 disorders is that while between large populations of
24 normal and abnormal individuals a correlation generally
25 can be determined for a particular biochemical, the
26 scatter that results from both analytical and biochemical
27 phenomena typically does not permit the level of a
28 particular biochemical to be utilized diagnostically for a
29 particular single individual. Nor may a single
30 biochemical value be utilized for the rational
31 prescription or development of a pharmaceutical for that
32 individual. This is not particularly surprising in that
33 both the levels and effects of a particular
34 neurotransmitter are modified by a number of other
35 neurotransmitters, in the same, or parallel metabolic
36 pathways. If, for instance, 5-HT (serotonin) is to be

1 used as a diagnostic tool for depression, suicidal
2 tendencies, or schizophrenia, it would be necessary and
3 perhaps provide a route to definitive diagnosis and
4 pharmaceutical specification or development, to
5 simultaneously determine the approximately 40 other
6 compounds that derive from tryptophan and significantly
7 effect the indolaminergic neuronal system's activity.

8 In recent years, LCEC (Liquid Chromatography with
9 Electrochemical Detection) has become a common tool for
10 the determination of catecholamines biogenic amines and
11 their metabolites in biological fluids. Because of
12 sensitivity limitations (typically 20-50 pg) and the
13 complexity of biological samples, both separation and
14 concentration steps typically have been necessary.
15 Heretofore, plasma catecholamine analysis typically
16 required three steps. First, the sample is collected and
17 the catecholamines separated and concentrated, for
18 example, using the alumina extraction procedure of Anton
19 and Sayre (See A.H. Anton and D.F. Sayre, J. Pharmacol,
20 Exp. Ther., 138 (1962), p. 360-375). The analytes,
21 norepinephrine, epinephrine and dopamine, along with the
22 internal standard DHBH (dihydroxybenzylamine), then are
23 separated chromatographically, and finally detected
24 electrochemically. Typical sample size requirements are
25 1.0 ml plasma or serum. In routine clinical use, there
26 have been numerous problems with conventional techniques
27 (alumina absorption, ion exchange and extraction), due to
28 a large number of poorly understood variables, in the
29 overall analysis system of sample acquisition, storage,
30 preparation and sensor response. These problems have
31 quite likely confused the relationships that may exist
32 between levels and distribution of the catecholamines and
33 various physiological and behavioral phenomena and disease
34 states.

35 In the analysis of complex biological materials such
36 as blood, serum and cerebrospinal fluids which may contain

1 numerous different constituents, the important (e.g.
2 abnormal) metabolites such as neurotransmitters to be
3 identified may be present in only parts per trillion.
4 While a chromatographic column can achieve macro
5 separation of the various constituents, it may not provide
6 adequate spatial (in time) separation of the extremely
7 small portion of metabolites of interest from the much
8 larger percentage of the many other compounds coeluted
9 from the column at the same time as the metabolites of
10 interest. Many of these interfering coeluted materials are
11 electrochemically active but electrochemically
12 irreversible, while many metabolites such as
13 neurotransmitters are both electrochemically active and
14 electrochemically reversible. It has been found that the
15 analytical problems of reliable measurements of
16 neurochemicals and related compounds are complicated by
17 the fact that interferences with conventional or prior
18 technologies are disorder related. This was discussed in
19 my prior publication, (Matson et al, Clinical Chemistry,
20 Vol. 30, No. 9, 1984) (see U.S. Patent 4,511,659) for
21 dopamine, dopac and serotonin measurements in directly
22 analyzed brain extract and cerebrospinal fluid for normal,
23 schizophrenics and Alzheimers. Recent work has indicated
24 that even for the widely used and accepted technique of
25 alumina extraction for plasma catecholamines that
26 inferences may be disorder specific. Higher values for
27 Norepinephrine (NE) and Epinephrine (EP) were observed
28 following alumina extraction and analysis of a single
29 energy conventional electrochemical detector than for a
30 three cell redox detector on neonatal stress blood
31 samples. Analysis of the neonate extracts on the sixteen
32 channel chemical imaging system revealed several
33 unexpected compounds that are potential interferences
34 including dihydroxyphenylacetic acid (DOPAC), 3
35 hydroxykynurenamine (3-OHKYA) and 3-hydroxy-anthranilic

1 acid (3-OHAN). These compounds have not been detected in
2 normal adult plasma alumina extracts.

3 In my aforesaid U.S. Patent No. 4,511,659, there is
4 provided an electrochemical detection system comprising a
5 plurality of coulometrically efficient electrochemical
6 cells, in series, for sequentially oxidizing and reducing
7 selected substances in a sample solution under controlled
8 conditions prior to measurement on a downstream testing
9 electrode or electrodes. More specifically, in accordance
10 with the invention provided in my aforesaid U.S. Patent
11 No. 4,511,659, a sample solution (e.g. a body fluid) is
12 passed through a suitable chromatographic column and the
13 eluant is streamed in contact with a series of
14 electrochemically isolated, in-line coulometric electrodes
15 operated under conditions so as to establish a series of
16 "gates" for the sequential oxidation and reduction of
17 substances in the sample solution whereby to screen
18 (remove) selected interfering and electrochemically
19 irreversible substances contained in the sample solution,
20 while passing selected electrochemically reversible
21 products for detection and measurement on a downstream
22 electrode. The gate electrode series is follows in-line
23 by one or more, preferably an array of six or more
24 coulometric measuring electrodes, each formed of porous
25 electrode base material such as fritted graphite, fritted
26 carbon or other conductive fritted material, for detecting
27 and measuring the electrochemically reversible compounds
28 of interest (e.g. neurotransmitters).

29 As reported in my aforesaid U.S. Patent No. 4,511,659,
30 there are several beneficial effects of this approach to
31 electrochemical analysis. Long-term drift in response is
32 effectively eliminated by acquiring essentially 100% of
33 the signal. The capability of analyzing essentially 100%
34 of a material allows the assay of compounds of unknown
35 purity by relating them to the basic principles of
36 electrochemical reaction embodied in Faraday's Law.

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1 Poisoning of the electrode, a dominant problem with
2 electrochemical sensors, is effectively eliminated by the
3 use of a much larger relative surface area for reaction.
4 And, finally, and most important to the eventual
5 development of array and gate cells, a coulometric
6 electrode by virtue of its essentially 100% efficiency
7 allows sequential oxidation and/or reduction of compounds
8 at successive-in-line detectors. The improved sensitivity
9 of the detection system as discussed in my aforesaid U.S.
10 Patent No. 4,511,659, particularly where two or more
11 active testing electrodes follow the screening electrodes
12 has given the ability to do direct injections of serum
13 filtrates and has also allowed the generation of
14 reproducible patterns of compounds with catecholamine like
15 electrochemical behavior of a large number of resolvable
16 components. This provides the possibility of performing
17 pattern recognition for the diagnosis or perhaps even
18 predictive diagnosis, of various disorders or disease
19 states.

20 In my copending application Serial No. 797,615 and its
21 parent U.S. Patent 4,863,873, I describe a system for
22 resolving and detecting hundreds of compounds in a single
23 sample at femtogram levels whereby to provide a small
24 molecule inventory or metabolic pathway pattern of an
25 individual. As taught in my aforesaid U.S. Patent
26 4,863,873, the small molecule inventory may be considered
27 to reflect the underlying activity and distribution of the
28 enzymatic pathways of an individual and hence reflect an
29 operational measure of the genome determining those
30 enzymes. The small molecule inventory of an individual may
31 thus be used to determine the health state of the
32 individual and/or to diagnose disease states. Correlation
33 of the patterns from a plurality of individuals provides
34 an understanding of the mechanisms of disorders or disease
35 states or conditions and, in turn, provides a rational
36 route to pharmacological development leading to treatment,

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1 cure or suppression of such disorders, disease states or
2 conditions.

3 The present invention is an improvement in the
4 invention described in my aforesaid U.S. Patent 4,863,873.
5 More particularly, in the practice of my invention as
6 described in my U.S. Patent 4,863,873, I have observed
7 that the biochemical analysis profiles of "normal" or
8 healthy individuals may vary quite widely, while the
9 biochemical profile analysis data of individuals having
10 disorders is far less chaotic. More particularly, I have
11 observed that the frequency distribution of certain
12 biochemical compounds or ratios of compounds in
13 individuals suffering from a disorder are far less chaotic
14 than "normal" or healthy individuals. This leads to a
15 general protocol for diagnosing, categorizing or
16 differentiating individuals based on comparisons of
17 biochemical analytical data of small molecule inventory
18 against data bases of known or previously diagnosed cases.
19 By way of example the process of the present invention may
20 advantageously be employed in the differentiation of
21 neurological degenerative dementing or affective disorders
22 such as Alzheimer's disease, Huntington's disease,
23 Parkinson's disease, schizophrenia, Progressive
24 Supernuclear Palsy, amyotrophic lateral sclerosis and
25 senile dementias from each other and neurologically normal
26 controls. Moreover, by suitable selection of variables,
27 the process of the present invention also is applicable to
28 classification of tumors, carcinomas, cardiovascular
29 abnormalities and other disorders. Similarly, the process
30 of the present invention advantageously may be utilized to
31 select therapy based on categories of known successful vs.
32 unsuccessful outcomes.

33 While not wishing to be bound by theory, the two
34 fundamental hypotheses underlying the process of the
35 present invention are:

1 1. The underlying genetic makeup or predisposition of
2 an individual will reflect through the proteins, enzymes,
3 and other factors it determines in patterns of small
4 molecules. Individual components within these patterns
5 will be affected by environmental effects such as diet,
6 stress or chemical inset; however, the overall pattern of
7 relationships will reflect the underlying operation of the
8 genome or the interference of a particular disorder. Among
9 the small molecules are the transmitters, cofactors and
10 metabolites that regulate neuronal and endocrine functions
11 and the interactions of somatic and central nervous system
12 processes. Thus, the compounds such as purines, tyrosine
13 and tryptophan derived neurotransmitters, peptides, pterin
14 and vitamin cofactors are highly relevant to the effect or
15 etiology of neurological disorders, cardiovascular
16 disfunction and certain tumors or carcinomas.

17 2. The relationships of these biochemical patterns
18 from a disorder are less chaotic or more regular than
19 those from healthy controls. All of the biochemical
20 systems of small molecules are interconnected and
21 interrelated in a complex web of feedback and response.
22 These interactions are highly nonlinear and thus,
23 depending on subtle differences in initial conditions, the
24 response of individual components in a biochemical pattern
25 will be highly variable. The overall system will thus
26 behave in a mathematically chaotic fashion. In a
27 disorder, elements within the biochemical pattern are over
28 or underregulated, thus reducing the degrees of freedom or
29 overall variability. Consequently, the presence of a
30 disorder implies more regulated or less chaotic
31 variability of compounds or relationships among compounds
32 in patterns from disordered individuals.

33 These two fundamental hypotheses provide an approach
34 to diagnostic categorization of disorders using frequency
35 distributions of compounds and relationships from large
36 data bases.

1 Description of the Drawings

2 For a fuller understanding of the nature and objects
3 of the present invention, reference should be had to the
4 following detailed description taken in combination with
5 the accompanying drawings wherein:

6 Figs. 1a-1d are frequency distribution graphs of
7 variables of Alzheimer's Disease and controls;

8 Fig. 2 is a geographical representation of scoring
9 algorithms;

10 Fig. 3 is a plot showing initial scoring of
11 Alzheimer's Disease v. control;

12 Fig. 4 is a plot similar to Fig. 3, with five
13 Alzheimer's Disease cases which scored as controls removed
14 from the Alzheimer's Disease scoring Distribution;

15 Fig. 5 is a plot showing analog distribution
16 measurements in accordance with the present invention;

17 Fig. 6A is a plot of measurements of nasal mucosa swab
18 samples at low gain;

19 Fig. 6B is a plot of nasal mucosa swab samples at high
20 gain; and

21 Fig. 7 is a plot of β -amyloid in saline suspension of
22 mucosa swab.

23 Detailed Description of the Invention

24 EXAMPLE I

25 Methodology for Sample Analysis and Data Base Creation

26 280 CSF samples from the categories Alzheimer's
27 Disease - AD (61 samples), Parkinson's Disease - PD (60
28 samples), schizophrenia - SC (60 samples), Huntington's
29 Disease - HD (20 samples), Supernuclear Palsy - PSP (13
30 samples) and neurologically normal controls - C (68
31 samples), were electrochemically analyzed in accordance
32 with the teachings of my aforesaid U.S. Patent 4,863,873.
33 Samples from normal and diseased individuals were prepared
34 and flowed through a chromatographic column, and detected
35 in a 16 sensor electrochemical cell using an NCA Chemical
36 Analyzer, Model No. CEAS available from ESA, Inc.,

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1 Bedford, Massachusetts. Sensor potentials ranged from T₁
2 -600mv to T₁₆ +900mv in 100mv increments. All samples
3 were from 7th or 8th mL aliquots of rostral caudal
4 gradients. Pools were created for each category utilizing
5 small subaliquots of the samples, and pools of all samples
6 were created for analytical quality control and evaluation
7 of unknowns. Samples were run under a variant of a
8 standard reverse phase gradient procedure in the
9 repetitive sequence Control Standard, Pool, 7 Samples,
10 Control Standard, Pool, ... as set forth in Table 1:

11 TABLE 1. CHARACTERISTICS OF METHOD 1.

12 Real time set up review; Times and events in the
13 methods chromatographic functions.

14 Review of live method: Potentials and currents of
15 channels 1-16 range functions temperature and limits.

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Table 1

REAL TIME SETUP REVIEW					
	TIME	DEVICE	FUNCTION	VALUE	TOTAL
1	0.00	FLOW	%B	5	1.20
2	0.20	CLEAN CELL	ON	960	
3	0.50	CLEAN CELL	OFF		
4	7.00	AUTO ZERO	ON		
5	7.20	FLOW	%B	5	1.20
6	7.58	AUTO-SAMPLER	INJECT		
7	8.00	FILE	START		
8	66.00	FLOW	%B	94	1.08
9	66.00	FLOW	%B	5	1.20
10	70.00	FILE	STOP		
11	70.00	FLOW	%B	5	1.20
12	70.00	AUTO-SAMPLER	STEP		
13	70.00	END		2	
14	70.00	METHOD	H147		
15					
16					
17					
18					
19					
20					

Review of Live Method									
Full Scale Current					P'stats				Autorange
10uA	100uA	10uA	10uA	10uA	-40mV	25mV	90mV	155mV	On
1uA	1uA	10uA	1uA	1uA	220mV	285mV	350mV	415mV	Floor
1uA	1uA	1uA	1uA	1uA	480mV	545mV	610mV	675mV	100nA
1uA	1uA	1uA	1uA	1uA	740mV	805mV	870mV	910mV	

Cell Box Temp : 35.0 C

			UPPER LIMIT	LOWER LIMIT
PUMP A:			350	0
PUMP B:			350	0
VALVE: Pos1				

8

Exit

Display Time Line

Mobile Phase:

A: 0.05 M LiPO₄, pH 2.86, 3 mg/L Dodecyl Sulfonic AcidB: 30% MeOH, 0.05 M LiPO₄, pH 2.86, 3 mg/L Dodecyl Sulfonic Acid

Column:

Dual 5u ODS, 4.6 mm x 15 cm ESA NBS

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1 Samples were analyzed for 38 known components (listed in
2 Table 2) and for 18 well-defined unknown peaks that were
3 isolated in all pools shown in Table 3. (Asterisks denote
4 components used in evaluation of regression and cluster
5 analysis statistical procedures for categorization of
6 groups.)

7 Table 2

8 Oracle compatible record showing retention times,
9 digitized characteristic responses across channels, and
10 set of control standard. Abbreviations are described in
11 Table 6.

12

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Table 2

Table: NUVASTD

Body: CSFRU01

Identified Compounds in Standard : 38

Missing Compounds in Standard : 0

#	File	Name		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	std0001	ASC	1.717	1.709	1.725	1.725	1.750	1.733											
	1.725	10000.00	3872279	9625125	1.27e+07	1.60e+07	1.50e+07	1.14e+07											
*2	std0001	CTS							2.267	2.267	2.250	2.267	2.275	2.283	2.292				
	2.283	1000.00							129467	251043	528682	1298734	1601456	2250767	1862584				
*3	std0001	URIC			2.333	2.342	2.350	2.350											
	2.350	0000.00			5672	1324197	3193191	1019120											
*4	std0001	XAN									3.042	3.033	3.050	3.067	3.067	3.100			
	3.050	1000.00									47171	1393667	3304089	2839171	350657	110609			
*5	std0001	IX														3.180	3.117	3.125	
	3.125	100.00														110609	154070	161130	
*6	std0001	VIA			4.883	4.867	4.875	4.875											
	4.875	10.00			387	4959	16674	13957											
*7	std0001	GSH							4.875	4.875	4.875	4.875	4.892	4.900	4.900				
	4.900	500.00							13957	28075	51532	117237	133772	197967	85618				
8	std0001	IE	5.625	5.642	5.642	5.650	5.650												
	5.642	10.00	334	18928	17463	1507	117												
*9	std0001	KMPG					6.075	6.075	6.075	6.075	6.083	6.092							
	6.075	10.00					749	13983	5545	1828	1718	1044							
10	std0001	HGA	6.392	6.400															
	6.392	10.00	16687	4976															
*11	std0001	G									7.942	7.942	7.950	7.950					
	7.942	100.00									2395	122100	53287	4420	975				
*12	std0001	GR										9.142	9.133	9.125	9.142	9.150	9.175	9.192	
	9.150	500.00										859	26350	194121	430593	1854244	189936	111915	
*13	std0001	LD	9.325	9.283	9.283	9.292	9.275												
	9.283	10.00	165	10336	14158	1566	562												
*14	std0001	BET										9.642	9.642	9.650	9.650	9.650	9.667	9.675	
	9.650	500.00										986	6461	26013	33540	107832	86595	31459	
15	std0001	AI			10.000	10.000	10.017	10.000											
	10.000	100.00			33745	118573	17512	2128											
16	std0001	EPI	10.167	10.142	10.150														
	10.142	10.00	367	14152	9783														
*17	std0001	I															10.250	10.217	
	10.217	100.00															26046	36349	
*18	std0001	DOPAC	11.825	11.833	11.833	11.842	11.850	11.833											
	11.833	10.00	247	7794	9749	1412	445	251											
19	std0001	3OHAN	13.225	13.200	13.217	13.242	13.250												
	13.200	10.00		1194	29905	8215	2107	1401											
*20	std0001	3OHIT	13.225	13.200	13.217	13.242	13.250												
	13.200	10.00		1194	29905	8215	2107	1401											
*21	std0001	4HBPA									13.442	13.450	13.467	13.450					
	13.450	100.00									13364	125434	43118	2233					
*22	std0001	4HB				13.683	13.692	13.683											
	13.692	10.00				1593	14700	3917											
23	std0001	4HBAC										13.983	13.992	13.992	14.000				
	13.992	200.00										75730	207495	311864	170423				
*24	std0001	TTR							15.250	15.242	15.225	15.233	15.250	15.250	15.292				
	15.233	1000.00							232	3866	102504	1101673	776764	117310	13964				
*25	std0001	5HTP			15.583	15.592	15.617												
	15.592	10.00			6994	16297	463												
26	std0001	DA		17.342	17.342	17.358	17.367	17.375											
	17.342	10.00		17722	6301	305	144	83											

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Table 2 (Cont'd.)

File	Name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
RT	Coac															
*27	std0001	SHIA	17.850	17.892	17.892	17.508	17.925									
	17.892	50.00	233	9330	119759	14584	549									
*28	std0001	HPAC						18.083	18.117	18.092	18.100	18.125				
	18.100	200.00						88	1143	37250	461257	287850				
*29	std0001	HTA											18.075	18.117	18.167	18.17
	18.117	50.00											59412	112086	54540	363
*30	std0001	30ND			21.575	21.608	21.608	21.608	21.592							
	21.608	12.00			50	2183	11535	2175	143							
*31	std0001	SHYL	22.167	22.200	22.200	22.217										
	22.200	10.00	47	1668	19401	1931										
*32	std0001	HTA			23.925	23.925	23.917	23.917	23.925	23.925	23.950					
	23.917	200.00			225	13994	237705	163964	47372	8852	2293					
*33	std0001	HTA										24.267	24.258	24.283	24.300	24.308
	24.258	100.00										181620	180990	55261	39928	21718
34	std0001	TTTA								24.475	24.492	24.517				
	24.492	80.00								2368	28935	5390				
35	std0001	SHL	30.742	30.783	30.792											
	30.792	10.00	231	6955	9627											
36	std0001	SHL			31.967	31.975	31.975	31.967	31.975							
	31.975	100.00			143	11501	33026	2425	171							
*37	std0001	TPOL						41.500	41.475	41.458	41.467	41.492				
	41.467	10.00						375	2006	7109	9905	4810				
*38	std0001	TBP			45.350	45.342	45.308	45.300	45.275	45.292	45.325	45.350	45.350			
	45.292	700.00			615	8371	23358	244497	967515	2056846	1090677	187013	109610			

Table 3. Unknown Pool Peak Matching.

Table shows dominant or maximum channel height and retention times of unknown peaks in the pool CSF with default concentrations set at 100. The peaks are referenced to XAN and Tyrosine, concentration 1000; HVA, concentration 200; Tryptophan, concentration 700.

The table shows the match of the seventh pool in the study to the nineteenth pool in the study and this represents typical drift over a two week period.

* Indicate variables used in regression analysis.

-11C-

Table 3

Sample: PCOL17
 Standard:
 Table: PCOL19A
 Study: CSF19A
 # Compounds identified: 22
 # Known Compounds Not Found: 0
 # Unknown Peak Clusters: 745

Compounds Identified

Compound	Conc	RT	RT Error	Height	Ratio Accuracy
<u>7/6</u>					
* p01	104.413971	2.617	0.100	167571	0.999
<u>10/11 12/11</u>					
XAN	973.750732	2.900	0.117	756026	0.861 0.959
<u>15/16</u>					
* p02	101.869156	3.108	0.125	208822	0.666
p03	131.687439	5.733	0.275	2747	
<u>13/14 15/14</u>					
p04	106.228569	8.242	0.325	142085	0.977 0.891
<u>9/10 11/10</u>					
TYR	979.260254	13.567	0.692	1375195	0.987 0.977
<u>13/14</u>					
* P05	99.553802	14.633	0.583	52515	0.943
<u>9/10 11/10</u>					
P09	89.990860	15.117	0.650	7632	0.850 0.923
<u>14/15</u>					
* P07	92.967072	15.275	0.600	55664	0.951
<u>10/11</u>					
P08	96.659180	16.133	0.417	29579	0.976
<u>9/10</u>					
* P10	96.603462	21.875	0.942	42516	0.949
<u>5/6 7/6</u>					
HVA	195.139450	22.475	0.800	30508	0.987 0.921
<u>16/15</u>					
P11	102.689194	24.892	0.717	16223	0.892
<u>9/10</u>					
P12	91.103188	25.150	0.850	10652	0.951
* p18	98.121086	26.850	1.308	470	
* p19	121.804512	27.192	1.142	324	
<u>12/13</u>					
P13	65.415916	28.158	0.883	53718	0.960
<u>13/14</u>					
P14	97.315338	29.542	1.108	24489	0.969
<u>9/10</u>					
P15	94.104630	29.642	1.100	10631	0.916
<u>10/11</u>					
P16	88.826569	32.425	0.767	2937	0.761
<u>13/14</u>					
P17	96.782722	37.992	1.133	22737	0.958
<u>9/10 11/10</u>					
TRP	674.284837	40.150	1.560	277033	0.970 0.955

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1 The analysis records were linked by a unique identifier to
2 clinical data of clinical diagnosis, diagnostic criteria,
3 age, pharmaceutical history, sex and race. Pools analyzed
4 as samples against standards for known values were
5 utilized to assess the precision of known compound values
6 in the data base. Standards analyzed against sequential
7 standards were used as a measure of instrumental
8 performance and pools analyzed against sequential pools
9 were utilized as a measure of the precision of unknown
10 peaks.

11 Validation of the Data

12 Control standards analyzed against sequential control
13 standards yielded precision values ranging from $\pm 1\%$ - $\pm 4\%$ CV
14 with no outlying values. Pools analyzed as samples gave
15 precision values ranging from $\pm 2\%$ - $\pm 7\%$ CV for compounds
16 present at the 0.5 ng/mL level or greater and typically
17 $\pm 25\%$ - 30% for compounds present at 2X the detection limit of
18 0.02-0.03 ng/mL (e.g. 5HT, EPI). Pools analyzed against
19 sequential pools for unknowns gave values of $\pm 3\%$ - $\pm 15\%$
20 coefficient of variation. Typically, the coefficient of
21 variation of the pools was 5-25 fold less than the
22 coefficient of variation of analytes in a group of
23 samples. Essentially, the contribution of assay
24 variability to the results is minimal.

25 The data base, upon completion, contained 280 samples
26 by 57 analytes (17,000 records). Of these, 163 were null
27 either because no peaks were detected at the sensitivity
28 limits of the assay, or because a signal detected did not
29 meet the qualitative criteria for purity.

30 Regression Analysis

31 Linear regression analysis and stepwise regression
32 analysis were used in a preliminary evaluation of the
33 data. Both raw and mean corrected data was evaluated.

34 Regression comparison of the AD group (61) vs.
35 controls (60) setting AD=1 and C=0 gave a categorical
36 separation regression equation with an S (standard error

-13-

1 of estimate) value=0.39 and $p=.0041$ for 27 of the most
2 significant known compound variables identified in
3 stepwise regression (labeled with asterisks in Table 2).
4 Inclusion of 7 of the most significant variables (labeled
5 with asterisks in Table 3) from the pool analyzed unknown
6 peak data base gave values of $S=0.382$ and $p=0.0037$.
7 Assuming a clinical diagnostic error rate in the order of
8 10%, seven AD samples with regression calculated values
9 (from -1.2 to 0.01) were removed from the calculation.
10 The regression characteristics were then $S=0.352$ and
11 $p=0.0031$.

12 Regression of the AD group with AD=1 vs. all others
13 (219)=0 for the same variable group yielded an equation
14 with $S=0.481$ and $p=0.0013$.

15 Observations: Although the AD group is separated from
16 mother groups with a high degree of probability, there is
17 too high a degree of overlap for a simple linear
18 regression algorithm to accurately categorize an
19 individual sample.

20 Cluster Analysis Procedures

21 Cluster analysis procedures using nearest neighbor and
22 furthest neighbor approaches were applied to the data
23 base. With both these approaches, the AD group tended to
24 cluster, but controls were scattered relatively evenly,
25 both outside and inside the AD region. Thus, the cluster
26 analysis approach is not suitable as a categorization tool
27 for this type of data.

28 Observations: The behavior of the data under cluster
29 analysis protocols, and the observations that the standard
30 deviations of compound values and of precursor/product
31 ratios across metabolic pathways within a disorder group
32 are smaller than within control groups is consistent with
33 the hypothesis that the biochemical response of controls
34 or normal individuals is more chaotic than that of
35 disordered individuals.

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1 Frequency Distribution Probability Analysis

2 The observations on the nature of the data
 3 distributions coupled with the technical ability to run
 4 large numbers of samples and variables offers an approach
 5 to categorization based on differences in the frequency
 6 distributions of variables in different disorder
 7 categories. This approach relies on basic probability
 8 considerations without any assumptions on the shape of a
 9 distribution curve or linearity of relationships.

10 The simplest question that I have investigated for the
 11 preliminary data base is that given an unknown sample,
 12 what is the probability (p) that that sample belongs in
 13 one group and not another.

14 For one variable, the question takes the form:

$$15 \quad \quad \quad f(V_1)_A \\ 16 \quad P = \frac{f(V_1)_A}{f(V_1)_A + f(V_1)_B} \\ 17 \\ 18 \\ 19$$

20 where $f(V_n)_A$ or $f(V_n)_B$ = the frequency with which an
 21 unknown sample value (V_n) occurs in category A or category
 22 B.

23 For multiple compounds, the expression expands:

$$24 \quad \quad \quad f(V_1)_A \cdot f(V_2)_A \dots f(V_n)_A \\ 25 \quad P = \frac{f(V_1)_A \cdot f(V_2)_A \dots f(V_n)_A}{f(V_1)_A \cdot f(V_2)_A \dots f(V_n)_A + f(V_1)_B \cdot f(V_2)_B \dots f(V_n)_B} \\ 26 \\ 27 \\ 28$$

29 If all frequencies are the same, the P value is 0.5 or
 30 a 50/50 chance that the unknown sample is A and not B. A
 31 positive answer compresses the expression to a 1 and a
 32 negative answer to 0.

33 Like cluster procedures and unlike regression, the use
 34 of the algorithm is independent of the number of variables
 35 used.

36 Implementation of the Procedure:

37 The implementation of the procedure is by the
 38 following steps:

39 1. Frequency distributions (shown in Figs. 1a-1d)
 40 were created by using a smoothing algorithm based on a 3

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1 point polynomial expansion function that treats each point
2 in the sparse data distribution with equal weighting as
3 the means of a distribution with a width at half height
4 proportional to its value. The use of smoothing functions
5 is a necessary assumption until the n of samples in a
6 particular category reaches approximately 300-400. The
7 procedure used was to divide all data in the categories by
8 the maximum value among categories X 85, apply the
9 polynomial expression algorithm, and normalize the data
10 distributions for the number of samples in each category.
11 The frequency distributions in each category are then
12 organized into look up tables for each variable (Table 4)
13 is then inserted into the look up table. Individual
14 values are divided by the range value X 85 and the
15 frequencies for each variable for category A and B are
16 sequentially calculated in the algorithm after subtracting
17 the effect of that sample from the frequency table. The
18 effect of a sequential calculation across a group of
19 variables listed and described in Table 6 is shown in
20 Figure 2 for 3 AD and 3 C cases from a group of 61 AD and
21 44 controls. One of the major features of the algorithm
22 is that no single variable predominates as a
23 differentiator among a large group of samples.

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1 TABLE 4. Distribution Table.

2 The table shows frequency distribution intervals 1-85
3 for two of the 71 variables in a look up table initially
4 used in scoring ad and control samples.

5 The tables are obtained by dividing all data by the
6 highest value for the combined data base times 85.

7 The raw distribution is then smoothed by a polynomial
8 expansion which treats each value with equal weighting as
9 the mean of a distribution whose scatter increases with
10 the value.

11 The overall distributions of groups are normalized to
12 the same area.

DISTRIBUTION TABLE

HVA			MHPG		
	AD	CONTROL		AD	CONTROL
1	0.010377	0.000205	1	1.2E-10	0.000000
2	0.045259	0.001702	2	0.000000	0.000003
3	0.126131	0.007945	3	0.000000	0.000028
4	0.242295	0.025793	4	0.000000	0.000140
5	0.379120	0.064431	5	0.000000	0.000576
6	0.503888	0.129976	6	0.000001	0.001932
.....
38	0.798418	0.938551	38	1.117459	0.742461
39	0.761982	0.889793	39	1.160736	0.801194
40	0.730955	0.836392	40	1.196534	0.855713
.....
83	0.006848	0.088021	83	0.225452	0.069938
84	0.005088	0.081885	84	0.208132	0.054130
85	0.003889	0.075893	85	0.190170	0.041177

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1 2. A sample record such as that shown in Table 5
 2 TABLE 5. DATA CASE RECORD.
 3 The data case record is spooled from the Oracle data
 4 base. In the example shown, the query requested
 5 biochemical data, the diagnosis "control" C and the I.D.
 6 "AT0022".
 7 The distribute range is created from the record by
 8 dividing each value by the maximum range value times 85 to
 9 indicate the look up range for the frequency of occurrence
 10 of the compound in the AD and C distributions.

Table 5

DATA CASE RECORD

	C AT0022	DISTRIBUT RANGE
MT3	0.018080	1
OHAN3	0.018080	3
OHKY3	0.090402	4
3CMD	1.970783	44
⋮	⋮	⋮
HVA	58.03809	56
MHPG	8.714764	62
⋮	⋮	⋮
12	2.911804	22
P01	57.25159	9
P02	135.8827	49
P03	1.808040	1
⋮	⋮	⋮

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1 TABLE 6. DESCRIPTION OF SCORING VARIABLES.

2 The table shows the arbitrary sequency in which 71
3 variables were applied to the initial scoring of AD vs. C
4 (See Figure 2).

5 The order of scoring has no effect on the final
6 outcome. Acetaminophen which was included in the assays
7 was not used as a scoring variable.

8 The table also contains the names of the known
9 compounds assayed and the probable moieties in the unknown
10 peaks inferred from the chromatographic/electrochemical
11 data and in vitro studies. Pathway ratios one to twelve
12 are calculated as molar ratios.

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Table 6

DESCRIPTION OF SCORING VARIABLES

<u>Variable Number</u>	<u>Abbreviation</u>	<u>Name or Possible Characteristic</u>
1	3MT	3 - Methoxytyramine
2	30HAN	3 - Hydroxyanthranilic Acid
3	30HKY	3 - Hydroxykynurenine
4	30MD	3 - O - Methyl dopa
5	4HBAC	4 - Hydroxybenzoic Acid
6	4HPAC	4 - Hydroxyphenylacetic Acid
7	4HPLA	4 - Hydroxyphenyllactic Acid
8	5HIAA	5 - Hydroxyindoleacetic Acid
9	5HT	5 - Serotonin
10	5HTOL	5 - Hydroxytryptophol
11	5HTP	5 - Hydroxytryptophan
12	A	Adenine
	AM	Acetaminophen
13	ASC	Ascorbic Acid
14	CYS	Cysteine
15	DA	Dopamine
16	DOPAC	Dihydroxyphenylacetic Acid
17	EPI	Epinephrine
18	G	Guanine
19	GR	Guanosine
20	GSH	Glutathione (reduced)
21	HGA	Homogentisic Acid
22	HVA	Homovanillic Acid
23	HX	Hypoxanthine
24	KYA	Kynurenic Acid
25	KYN	Kynurenine
26	LD	L-Dopa
27	MET	Methionine
28	MHPG	Methoxy-Hydroxyphenyl Glycol
29	NE	Norepinephrine
30	NMN	Normetanephrine
31	TPOL	Tryptophol
32	TRP	Tryptophan
33	TYR	Tyrosine
34	TYRA	Tyramine
35	URIC	Uric Acid
36	VMA	Vanillylmandelic Acid

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Table 6 (Cont.)

<u>Variable Number</u>	<u>Abbreviation</u>	<u>Name or Possible Characteristic</u>
37	XAN	Xanthine
38	P01	Methoxyhydroxybenzene -
39	P02	Cysteine or Methionine Di Peptide
40	P03	Catechol -
41	P04	Catechol -
42	P05	Cysteine Condensation or Peptide
43	P07	Cysteine Condensation or Peptide
44	P08	TRP or TYR Peptide
45	P09	TRP or TYR Peptide
46	P10	Indole -
47	P11	Indole -
48	P12	Methoxybenzene -
49	P13	Indole -
50	P14	Hydroxybenzene -
51	P15	TYR or TRP Peptide
52	P16	TYR or TRP Peptide
53	P17	Indole -
54	P18	Hydroxyindole -
55	P19	Hydroxyindole -
56	HVA_A	Oxidative of Backwave of HVA
57	TPR_A	Oxidative of Backwave of TRP
58	TYR_A	Oxidative of Backwave of TYR
59	XAN_A	Oxidative of Backwave of XAN
60	1	TRP/5HIAA + 5HTOL + 5HT + 5HTP
61	2	TRP/OHAN + KYN
62	3	TRP/KYN + HTP + HTOL + 5HIAA + 5HT
63	4	HTP/5HIAA
64	5	5HIAA/5HTOL
65	6	KYN/OHKY
66	7	HVA/5HIAA
67	8	TYR/HPLA
68	9	TYR/HVA + LD + E + NE + MHPG + DA + 3MT + NMN
69	10	HVA/MHPG
70	11	TYR/HPLA + HVA + LD + E + NE + MHPG + DA + 3MT + NMN
71	12	XAN/HVA

1 Testing the Algorithm on AD vs. Controls

2 For an initial test, conditions were set up such that
3 each individual sample was evaluated as if the data base
4 were set up without its contribution. The results of the
5 initial scoring are shown in Fig. 3. The scoring of five
6 of the 61 AD cases as controls (p =less than 0.01 that the
7 sample is an AD and not a control) is not surprising given
8 the probable diagnostic error rate in AD. The scoring of 4
9 of the controls as AD are of concern.

10 One possible explanation is that the AD data base is
11 in effect contaminated by five cases that clearly do not
12 match the overall AD profile and are probably not AD.
13 When these five samples are removed from the AD data base
14 and all samples, including the 5 removed, are scored, the
15 control and AD groups are uniquely separated as shown in
16 Fig. 4. The five samples that were removed from the AD
17 scoring data group distribute in an equivocal region from
18 0.1 to 0.9. In subsequent application of the procedure
19 and algorithm to AD samples vs. all other samples (PD, SC,
20 HD, PSP and C) in the data base asking the question is
21 this sample in the AD distribution and not in the
22 distribution of all others yielded similar results scoring
23 AD samples with p values = 0.98 or greater. The
24 distribution of scores of all others was scattered from
25 0.001 to .8 including the 5 AD samples which previously
26 scored in this region vs. controls.

27 Testing PD vs. Controls and PD vs. AD

28 Eighteen unknown peaks were initially selected for
29 assay in all samples because of differences among
30 preliminary pools of AD, PC, C and SC. Of these, six
31 occurred only in one sample of the group and 11 had
32 distributions of only slight differentiation capability.
33 One peak, designated P05 shown in Figure 5, occurred
34 predominantly in the PD group. Otherwise, values above
35 100% of pool values were seen in only six of the AD group.
36 The mean values of P05 and S.D. relative to a pool value

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1 of 100 were PD 415 ± 200 , Control 20 ± 15 , AD 30 ± 80 , and
2 SC 28 ± 17 . This one variable utilized alone in the
3 distributin p scoring algorithm separates PD from controls
4 with PD values from 0.4 - 0.999 and C values from 0.51 -
5 0.001. With all variables, the PD values were all greater
6 than 0.994 and C values less than 0.003.

7 Scoring AD and not PD without P05 separated 58 PD
8 samples with scores of less than 0.02 and two with scores
9 of 0.14 and 0.18. 55 AD samples scored above 0.98 with
10 six in the region between 0.25 and 0.76, including the 5
11 cases which initially scored as controls. Including P05
12 in the scoring, all PD samples scored below 0.001. 57 of
13 the AD samples scored above 0.98 and 4 of the initial 5
14 that matched the control group scored near the PD samples
15 from 0.11 to 0.32.

16 Example II

17 Pathological Changes in the Olfactory System 18 in Alzheimer's Disease

19 In Alzheimer's Disease a number of studies have shown
20 that the structures of the limbic system, including
21 entorhinal cortex, hippocampal formation, basal forebrain
22 and the amygdala are the most severely affected areas of
23 the brain. Since the amygdala, entorhinal cortex and
24 uncal hippocampus are strongly related to olfactory input
25 (Pearson et al, 1985) ¹ suggested that the olfactory
26 pathways may be the initial site of pathology in the
27 disease. In fact some authors have suggested that inhaled
28 molecules, such as aluminosilicates, could contribute to
29 the etiology of the disease (Roberts, 1987).²

30

31

32 1. Pearson R.C.A., Esiri M.M., Hiorns R.W., Wilcock G.K.
33 Powell T.P.S. Anatomical correlates of the distribution
34 of the pathological changes in the neocortex in
35 Alzheimer's disease. Proc. Natl. Acad. Sci. 82:
36 4531-4524, 1985

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38 2. Roberts, E. Alzheimer's disease may begin in the nose
39 and may be caused by aluminosilicates. Neurobiol.
40 Aging. 7:561-567, 1986; Shipley M.T. Transport of
41 Footnote Cont. Next Page

1 The olfactory nerve cells in the olfactory epithelium
2 project through the cribiform plate to the olfactory bulb
3 (Kosel et al, 1981)³ Primary olfactory fibers synapse in
4 olfactory glomeruli with descending dendrites of mitral
5 and tufted cells; which are the primary output neurons of
6 the bulb. Axons of mitral and tufted cells enter the
7 olfactory tract and provide input to the anterior
8 olfactory nucleus, as well as to the central projections
9 of the olfactory system. The anterior olfactory nucleus,
10 located in the center of the bulb, gives rise to a
11 recurrent collateral to the bulb and to a crossed
12 projection to the anterior commissure. The olfactory
13 tract passes through the anterior perforated substance and
14 projects to prepiriform cortex, corticomедial nuclei of
15 the amygdala, entorhinal and perirhinal cortices, inferior
16 surface of the frontal lobe, insula, temporal pole and
17 basal forebrain (Haberty et al, 1977).⁴

18 Several lines of evidence have shown that the
19 olfactory system is affected in Alzheimer's Disease.
20 Several groups have shown that Alzheimer's Disease
21 patients show deficits in olfactory recognition (Doty et
22 al, 1987).⁵ A study which examined patients with very

23
24
25 Footnote 2 Cont.

26 molecules from nose to brain; transneuronal anterograde
27 and retrograde labeling in the rat olfactory system by
28 wheat germ agglutinin-horseradish peroxidase applied to
29 the nasal epithelium. Brain Res. Bull. 15: 129-142,
30 1985; Peri D.P., Good P.F. Uptake of aluminum into
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38 4. Haberty L.B., Price J.L. The axonal projection
39 patterns of the mitral and tufted cells in the olfactory
40 bulb in the rat. Brain Res. 129: 152-157, 1977.

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42 5. Doty R.L., Reys P.F., Gregor T. Presence of both odor
43 identification and detection deficits in Alzheimer's

44 Footnote Cont. Next Page

1 mild Alzheimer's Disease showed impairment on a task of
2 identification of odors, however olfactory thresholds were
3 normal (Koss et al, 1988).⁶ Pathologic studies have
4 recently documented changes in the primary olfactory
5 receptor neurons in Alzheimer's Disease. Talamo et al,
6 1976⁷ reported nasal epithelium from Alzheimer's Disease
7 patients showed increased immunoreactivity for
8 phosphorylated neurofilaments, as well as Tau and Alz-50
9 positive neurites. These neurons however did not contain
10 neurofibrillary tangles. Neurite formation in the
11 olfactory epithelium did correlate with numbers of
12 neurofibrillary tangles and senile plaques in the
13 hippocampus of the Alzheimer's Disease brains. The
14 possibility of using biopsy of nasal epithelium as a
15 clinical marker of Alzheimer's Disease during life was
16 suggested.

17 Pathological studies of the olfactory bulb in
18 Alzheimer's Disease have shown consistent changes. Esiri
19 and Wilcock, (1984)⁸ found neurofibrillary tangles in the
20 olfactory bulb in tufted cells, outer granule cells, and

21
22
23

24 Footnote 5 Cont.

25 Disease. Brain Res. Bull. 18: 597-600, 1987; Green
26 J.E., Songsanand P., Corkin S., Growdon J.H. Olfactory
27 capacities in Alzheimer's Disease. Neurology 39, Suppl
28 1: 138, 1989; Rezek D.L. Olfactory deficits as a
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33 Olfactory detection and identification performance are
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37 7. Talamo B.R., Rudel R.A., Kosik K.S., Lee V.Y.M., Neff
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42 8. Esiri M.M., Wilcock G.K. The olfactory bulbs in
43 Alzheimer's Disease. J. Neurol. Neurosurg. Psychiatr.
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1 in the anterior olfactory nucleus. Hyman and colleagues
2 (1991)⁹ has examined 10 control and 10 Alzheimer's
3 Disease olfactory bulbs. Large numbers of neurofibrillary
4 tangles were consistently found in the Alzheimer's Disease
5 anterior olfactory nucleus, with only small numbers in the
6 mitral and tufted cells. This is consistent with other
7 primary sensory systems in Alzheimer's Disease in which
8 higher order association areas show more severe pathology
9 than primary sensory areas.

10 The olfactory epithelial neurons and olfactory bulb
11 have been shown to contain very high levels of carnosin
12 (β -alanyl-L-histidine), and several studies have shown
13 that carnosine is released in the olfactory bulb in
14 response to peripheral inputs (Macrides et al, 1983).¹⁰
15 The external tufted cells in the olfactory bulb contain
16 dopamine, substance P or both. The deeper tufted cells
17 may use excitatory amino acids. Periglomerular cells
18 contain GABA, enkephalin or dopamine. The olfactory bulb
19 receives a very strong centrifugal projection including
20 enkephalin, vasoactive intestinal polypeptide, LHRH,
21 somatostatin and substance P fibers. A cholinergic
22 projection originates mainly in the ventral nucleus of the
23 diagonal band. Serotonergic projections originate from
24 the dorsal raphe nuclei, while there is a noradrenergic
25 projection from the locus ceruleus. The olfactory bulbs
26 therefore receive a rich and variegated neurochemical
27 input, including projections from the cholinergic,
28

29

30

31 9. Hyman B.T., Arriagada P.V., Van Hoesen G.W.
32 Pathological changes in the olfactory system in aging
33 and Alzheimer's disease. Int. Study Group for the
34 Pharmacology of Memory Disorders, in press. (1991)
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36 10. Macrides F., Davis B.L. The olfactory bulb. In:
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1 noradrenergic and serotonergic nuclei which are known to
2 be affected in Alzheimer's Disease (Macrides et al, 1983).¹¹

3 Several factors relating to the suitability of using
4 nasal secretions for neurochemical analysis have been
5 examined. These include sample acquisition, potential
6 effects of bacterial or viral infection, the specific
7 compounds which can be identified, and means for
8 normalizing the data to compensate for sampling
9 variability and sample size.

10 Following the electrochemical analysis procedure above
11 described for CFS all the compounds listed in Table 2 have
12 been identified using nasal secretion samples although the
13 concentration profiles are significantly different than
14 for CSF. (See typical patterns in Figures 6A and 6B.)
15 Notably, dihydroxyphenylacetic acid (DOPAC) is present in
16 greater concentrations than homovanillic acid (HVA) and
17 serotonin (5HT) is relatively equivalent to
18 5-hydroxyindoleacetic acid (5HIAA). Also notably different
19 is the complexity of the region of small di- and tri-
20 peptides with retention times of 30-45 min on channels
21 10-15. Essentially, the nasal mucosa appear to contain
22 representative compounds from all metabolic pathways that
23 occur in the neuronal projections to the olfactory bulb.

24 Two characteristics make nasal secretions attractive
25 for neurochemical analysis. The secretions are close to
26 the site or origin of the compounds being measured and
27 they are in a strong reducing environment. Oxidation
28 potential measurements of saline suspensions of nasal
29 secretions with platinum vs. Ag/Ag/Cl yield values from
30 -0.230 to -0.300 mV compared to lumbar CSF values of
31 -0.050 to -0.170. The reducing character of the tissue is
32 consistent with observation of a large number of easily

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36 11. Macrides F., Davis B.L. The olfactory bulb. In:
37 Emson P.C. (ed.) Chemical Neuroanatomy, Raven Press,
38 New York, pp. 391-426, 1983

1 oxidized peaks on the lower channels of the
2 chromatographic pattern. The reducing character of the
3 sample makes it highly attractive for clinical use because
4 of the stability inferred for the target compounds.
5 Indeed preliminary studies indicate no change in
6 5-hydroxyindoleacetic acid or homovanillic acid over 24
7 hours in perchloric acid extracts at room temperature.
8 From the number of peaks in the 35-50 min region, the
9 nasal mucosa also appear to be a stable matrix for small
10 peptides, including fragments of β -amyloid peptide. This
11 hypothesis was tested by incubating β -amyloid in saline
12 suspensions of nasal mucosa and observing the development
13 over 6 hours of electroactive peaks, five of which
14 corresponded to original peaks in the suspension as shown
15 in Figure 7.

16 Sample Acquisition Preparation and Normalization

17 Samples typically weigh approximately 100 mg. A
18 preliminary evaluation of swabs taken from high in the
19 nostril, approximately 2 cm, vs. a wipe of the nares at
20 approximately 0.5 cm, indicate that all species of
21 interest are present lower in the nose, but at
22 significantly reduced concentrations overall. In an
23 initial study from an n of 4 there were no significant
24 changes in the ratio of DOPAC/HVA or in tryptophan (TRP)
25 to kynurenine (KYN), but the ratio of 5HIAA/5HT increased,
26 consistent with reduced concentrations of 5HT in swabs
27 obtained lower in the nose. Similarly, on an n of 4, a
28 relatively aggressive scrubbing the swab yields a pattern
29 with higher concentrations than a more gentle scrub, but
30 without significant changes in overall pattern.

31 Swabs were extracted by cutting the tip and placing it
32 in 300 μ l of 0.1 M HClO₄, they ere vortexed for 1 min.
33 centrifuged and then reextracted in another 300 μ l of 0.1
34 M HClO₄. Further sequential extractions did not result in
35 any improvement in recoveries. Following centrifugation
36 the pellets are combined, and can be extracted with 40:60

1 acetonitrilehexane for analysis of ubiquinones and larger
2 peptides.

3 Several brands of cotton swab were evaluated for blank
4 effects. Of these, plastic handled Johnson and
5 Johnson (TM) swabs proved clean enough to be used as
6 received. Paper composite and wooden handles of various
7 types showed a number of small peaks that have the
8 potential to interfere with the target compounds of
9 interest.

10 The selection of appropriate divisors or normalizers
11 of the data is a major consideration since sample size is
12 not controllable. This issue was investigated in a
13 preliminary study of 6 individuals from whose left and
14 right nostril swabs were obtained. From the initial data
15 analysis, xanthine (XAN), uric acid (UA), tyrosine (TYR),
16 DOPAC, HVA, TRP, KYN, 5HIAA and 5HT have been measured.
17 The 6 left and right individual samples as duplicate pairs
18 coefficient of variation were from ± 60 - $\pm 80\%$. When all
19 data was divided by xanthine, the variations were ± 15 -
20 $\pm 30\%$. When precursor product ratios were used, the
21 variations were further lowered (DOPAC/HVA mean $1.4 \pm 7\%$;
22 TRP/KYN mean $2.6 \pm 14\%$; and 5HT/5HIAA mean $1.1 \pm 13\%$)

23 Possible Effects of Bacterial and Viral Infections

24 Two available cultures of Pseudomonas and
25 Staphylococcus were evaluated to look for possible
26 interferences from bacterial infection. Aliquots of
27 approximately 10 mg wet weight of cultured bacteria
28 isolated by centrifugation and washing were sonified in
29 200 μ L of 0.1 M HClO₄, centrifuged and the supernatant
30 analyzed. Responses for XAN, KYN, TYR, TRP, guanosine and
31 hypoxanthine were observed of approximately 50-100% of the
32 response of a typical nasal swab. No dopaminergic,
33 serotonergic, or noradrenergic metabolites were observed.
34 Consequently, it is thought that bacterial contamination
35 would have to be quite massive, constituting over 10% of a

1 typical sample to have a 10% or greater effect on the
2 measurements.

3 In the initial study three of the six subjects had
4 colds and there were no significant differences between
5 them and unaffected individuals. There was a slight, but
6 not statistically significant increase in KYN/XAN and a
7 decrease in TRP/KYN ratios.

8 It thus appears that nasal secretions may
9 advantageously be employed as samples for neurochemical
10 analysis using electrochemical detection techniques.
11 Similar results were found using platelets.

12 Moreover, the invention advantageously may be employed
13 for diagnosing a disease condition at an early stage, i.e.
14 before observable physical manifestations. For example,
15 in the case of Alzheimer's Disease, the exact etiology is
16 unknown. However, there is strong evidence that genetic
17 factors play a role (St. George-Hyslop et al, 1990).¹²
18 The means by which a genetic defect contributes to the
19 pathologic features of the illness is unclear. A major
20 feature of the pathology is the accumulation of the
21 β -amyloid protein in senile plaques, blood vessels, skin

22 By correlating changes in neurochemical markers with
23 changes in accumulation of the β -amyloide protein as the
24 Alzheimer's Disease progresses, it is possible to provide
25 an early diagnosis for Alzheimer's Disease. Also,
26 identification of neurochemical markers for Alzheimer's
27 Disease may provide a basis for prevention and/or
28 treatment, i.e. by identifying precursors, progression of
29 the disease may be slowed, stopped or even reversed.

30 The invention has been described for use in diagnosing
31 Alzheimer's Disease. It will be understood, however, that
32 the invention advantageously may be used to diagnose and

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35 12. St. George-Hyslop P.H., Haines, J.L., Farrer L.A., et
36 al. Genetic linkage studies suggest that Alzheimer's
37 disease is not a single homogeneous disorder. Nature
38 347: 194-197, 1990.

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1 characterize other neurological, degenerative or defective
2 disorders such as Huntington's Disease, Parkinson's
3 Disease, schizophrenia, progressive supernuclear palsy,
4 amyotrophic lateral sclerosis and senile dementias. The
5 invention also advantageously may be used to classify and
6 diagnose tumors, carcinomas, cardiovascular abnormalities
7 and other disorders, or for selection of therapy based on
8 categories of known successful vs. unsuccessful outcomes.
9 Moreover, both treatment protocols and new pharmaceuticals
10 may be evaluated.

11 Still other changes and advantages will be obvious to
12 one skilled in the art.

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16 13. Joachim C.L., Morris J.H., Selkoe D.J. Clinically
17 diagnosed Alzheimer's disease: autopsy results in 150
18 cases. Ann. Neurol. 24: 50-56, 1988.

CLAIMS

1. In a method for diagnosing disorders in which biological samples from normal and afflicted (abnormal) individuals are analyzed to generate patterns representative of molecular constituents of said samples, the improvement which comprises creating a data base of frequency distribution patterns of constituents of samples from organisms having known categories of disorders and controls, and comparing the sample analysis for conformity to said frequency distribution patterns.
2. A method according to claim 1, wherein said samples comprise body fluid.
3. A method according to claim 2, wherein said body fluid is selected from cerebrospinal fluid, plasma, platelets, nasal mucosa, serum, saliva, and urine.
4. A method according to claim 1 or 2 or 3, wherein said samples comprise electrochemically active compounds, and including the steps of passing said fluid samples sequentially through a liquid chromatographic column for achieving time-space separation of the materials eluting from the column and an electrochemical detection apparatus whereby to generate electrochemical patterns of said electrochemically active compounds.
5. A method according to claim 1 or 2 or 3 or 4, wherein said disorder is selected from Alzheimer's Disease, Parkinson's Disease, Huntington's Disease, schizophrenia, Progressive Supranuclear Palsy, amyotrophic lateral sclerosis, senile dementia, tumors, carcinomas, and cardiovascular abnormalities.
6. A method according to claim 4, and including the step of separating said electrochemically active compounds by electrochemical characteristics in said electrochemical detection apparatus.
7. A method for diagnosing disorders in which biological samples from normal and afflicted (abnormal) individuals are analyzed to generate patterns

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representative of selective parameters, the improvement
5 which comprises examining said patterns for chaotic or
non-linear values.

8. A method according to claim 7, wherein the
parameters measured comprise biochemical patterns of
small molecules.

9. A method according to claim 8, wherein said
parameters are representative of at least one of the
following selected from the group consisting of
neurotransmitters, cofactors, precursors, metabolites,
5 and associated compounds.

10. A method according to claim 7, wherein said
disorder comprises Alzheimer's Disease, and the parameters
measured comprise tyrosine and tryptophan peptide
degradation fragments from beta amyloid.

11. A method according to claim 7, wherein said
disorder comprises Parkinson's Disease, and the parameter
measured comprises the amylyte designated P05.

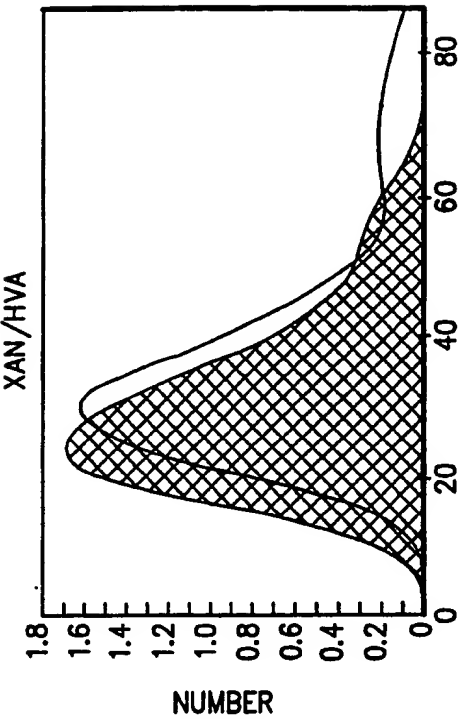


FIG. 1b

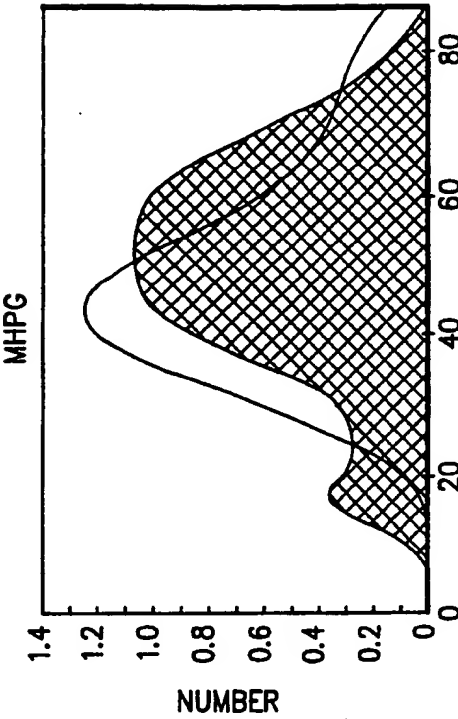


FIG. 1d

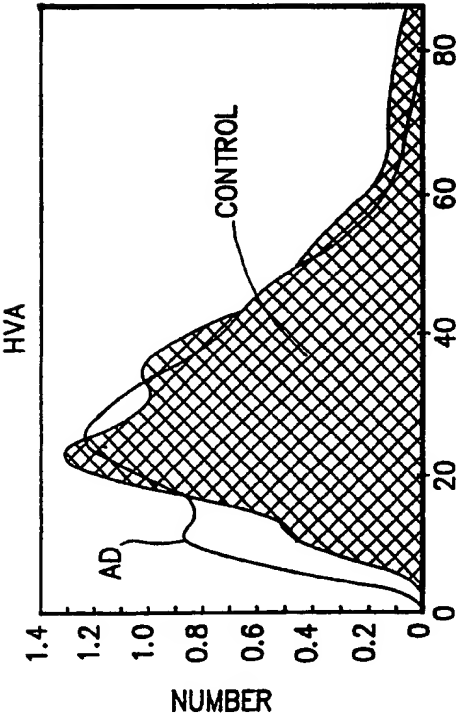


FIG. 1a

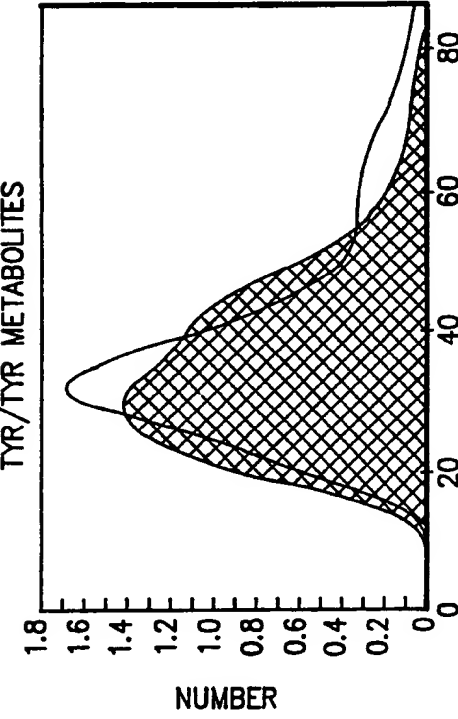
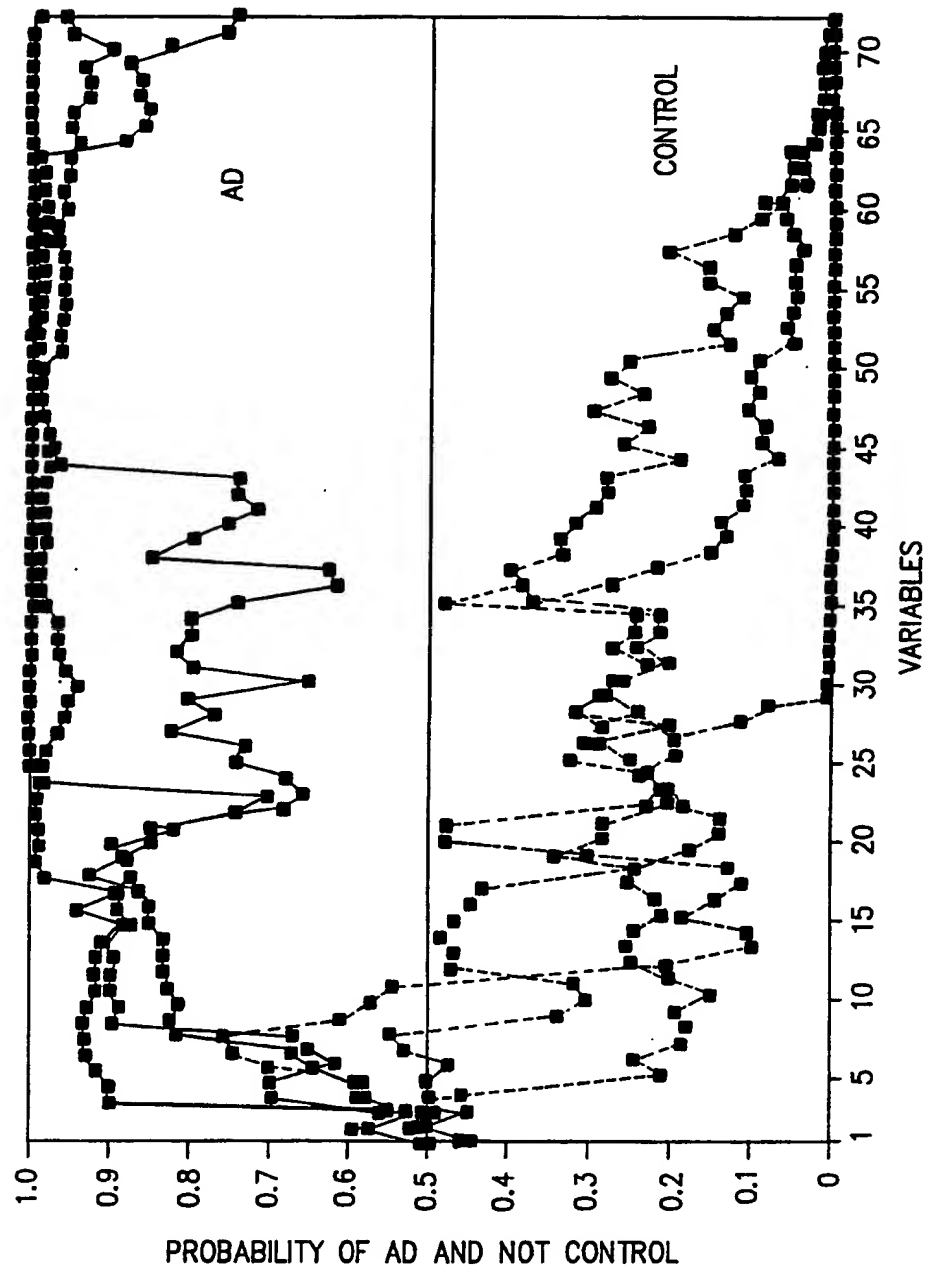


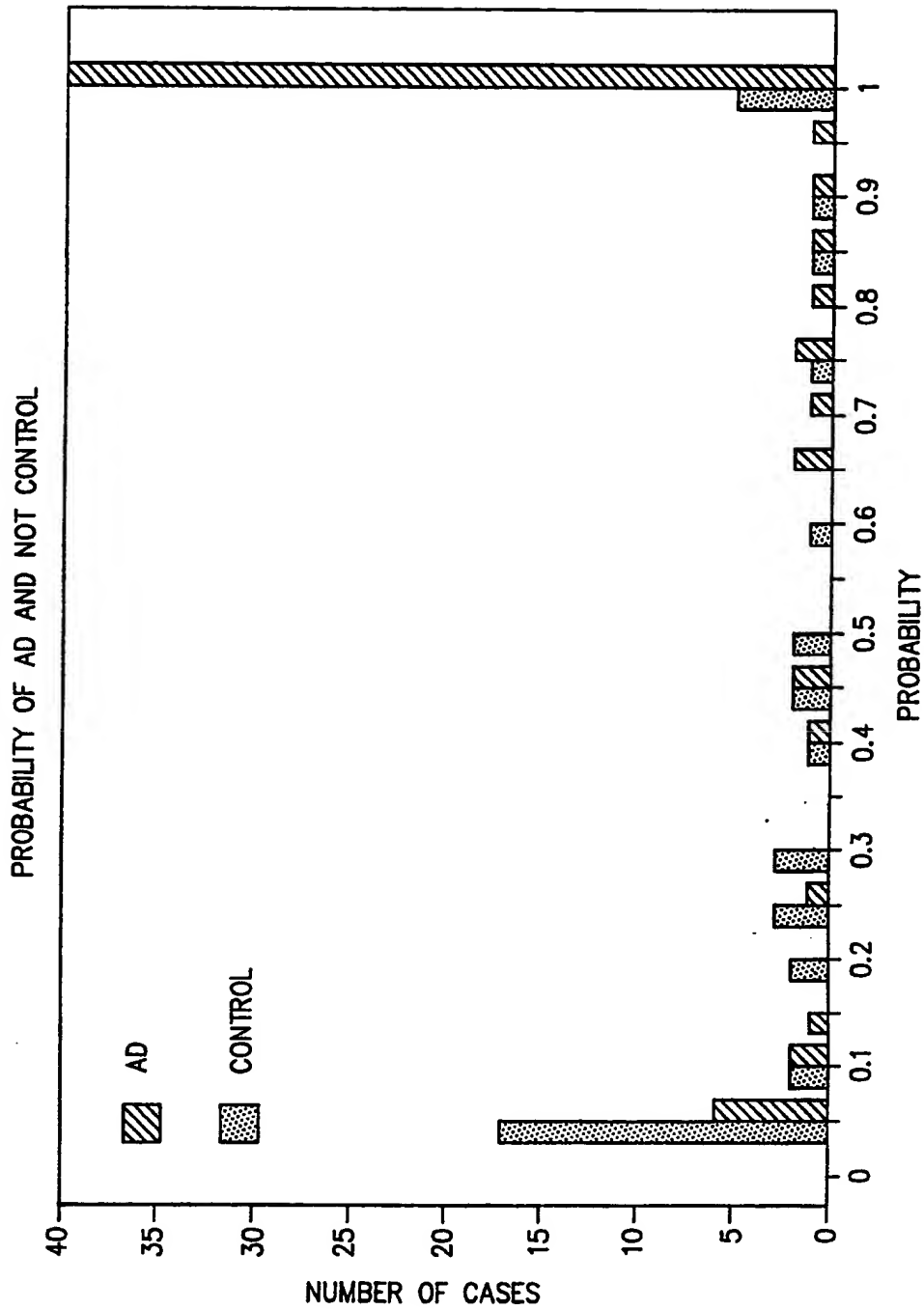
FIG. 1c

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FIG.2

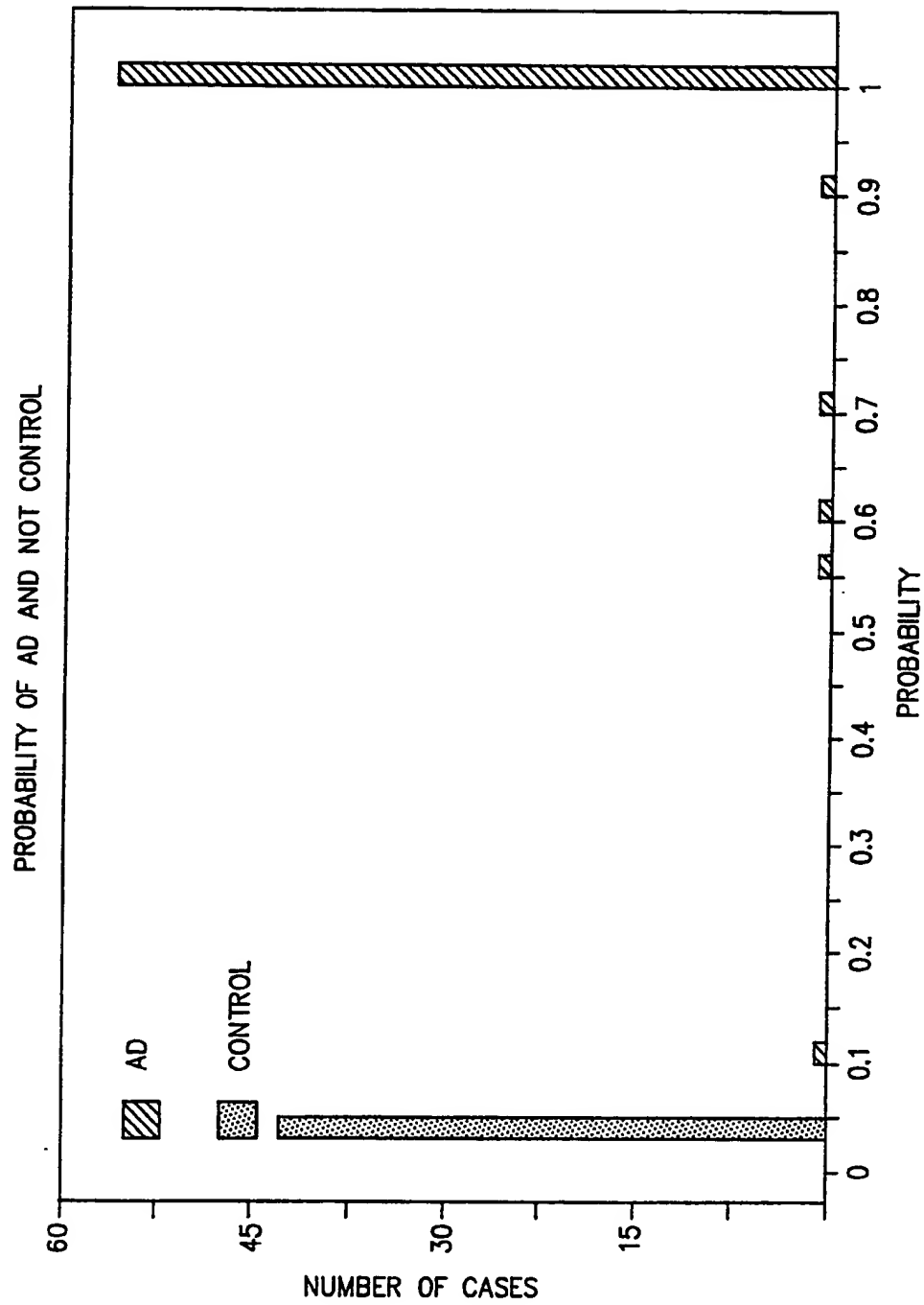
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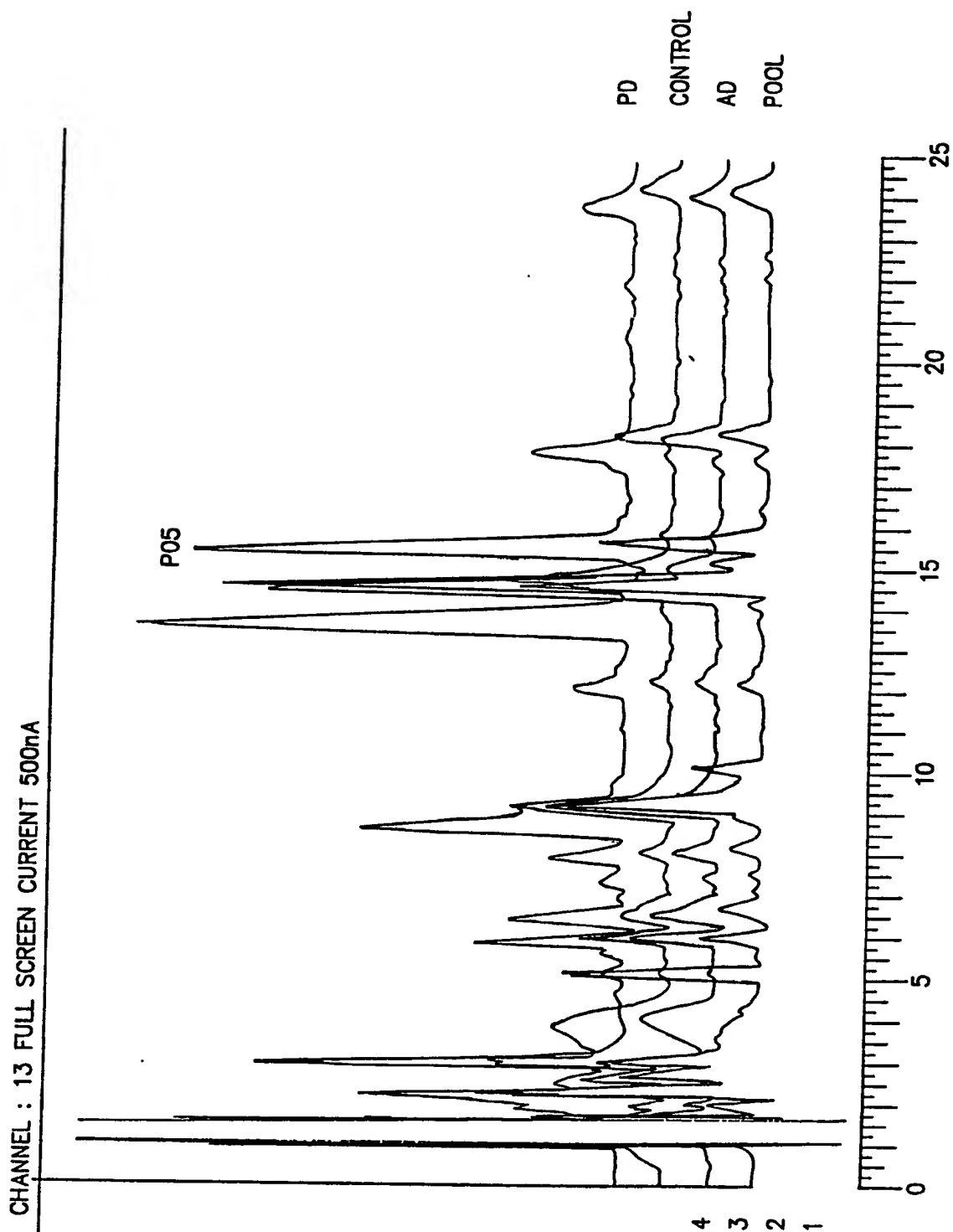
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**FIG. 4**

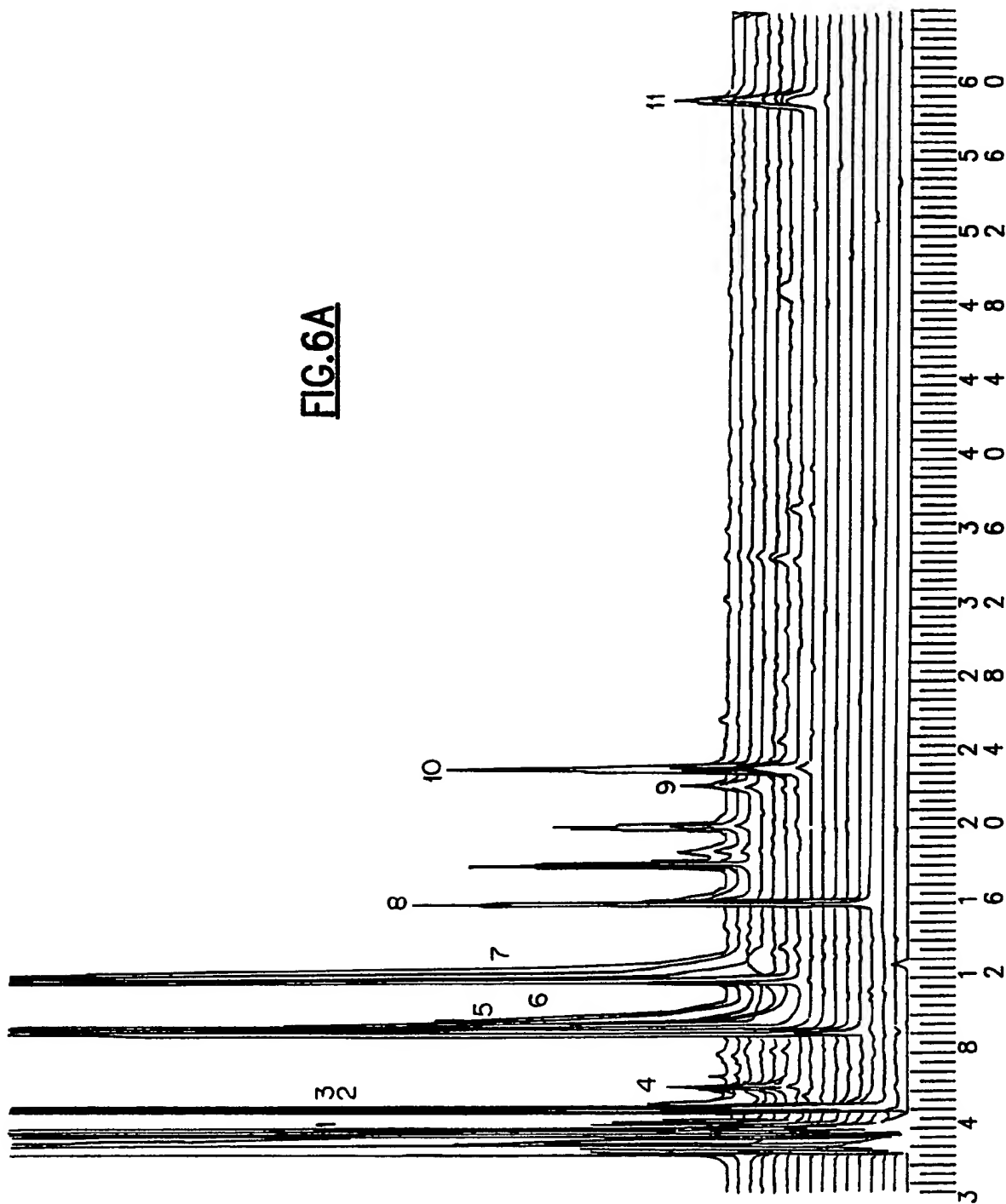
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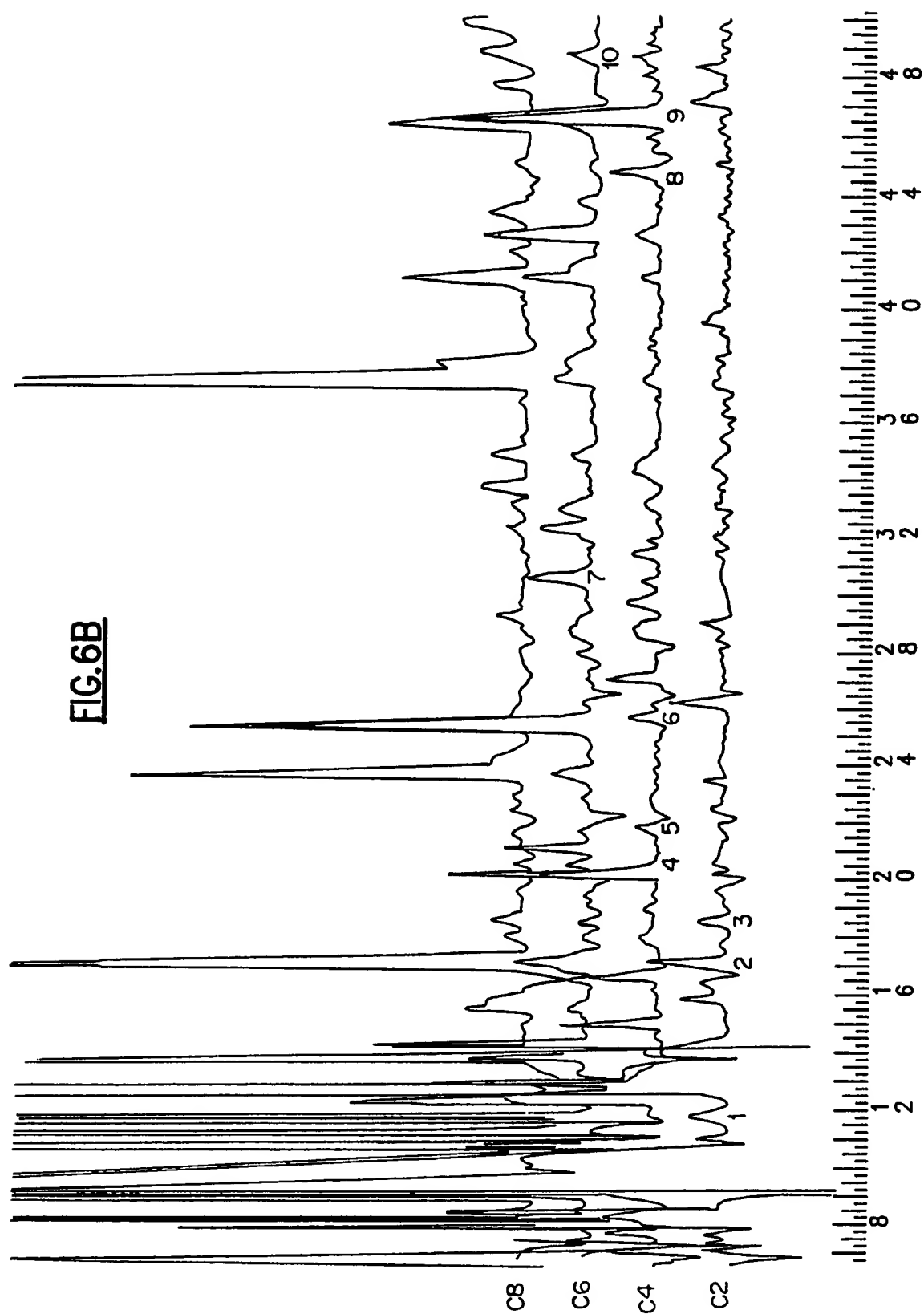
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FIG. 5

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FIG. 6A

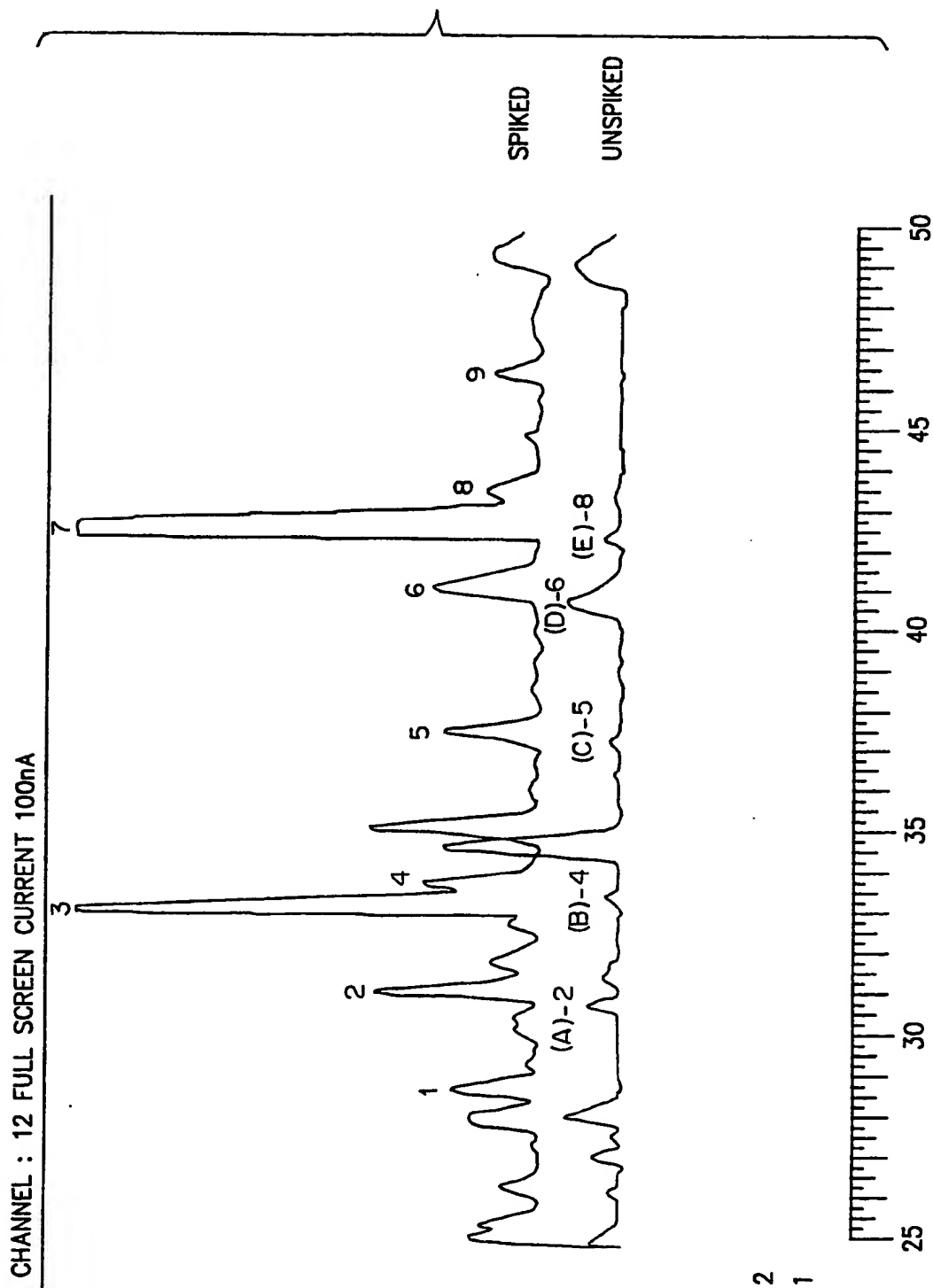




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FIG. 7



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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 92/00375

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC Int.Cl.5 G 01 N 33/48 G 01 N 27/28 G 01 N 27/416 G 01 N 30/64 G 01 N 33/68		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl.5	G 01 N	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	WO,A,8603006 (ESA, INC.) 22 May 1986, see the entire document, & US,A,4863873 (cited in the application) ---	4-11
X	EP,A,0371517 (ESA, INC.) 6 June 1990, see the entire document, & US,A,4511659 (cited in the application) ---	4-11
A	E.J. TAYLOR: "Dorland's Illustrated Medical Dictionary", 27th edition, 1988, W.B. Saunders Co., Philadelphia, PA, US, see page 461: "Diagnosis" -----	4-11
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>⁹ Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
20-05-1992		24.06.92
International Searching Authority		Signature of Authorized Officer
EUROPEAN PATENT OFFICE		Nicole De Bie

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**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9200375
SA 56291

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 18/06/92. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 8603006	22-05-86	AU-A- 5202186	03-06-86
		EP-A- 0201591	20-11-86
		JP-T- 62500805	02-04-87
		US-A- 4863873	05-09-89

EP-A- 0371517	06-06-90	US-A- 4511659	16-04-85
		CA-A- 1195383	15-10-85
		EP-A, B 0122009	17-10-84
		JP-A- 60022654	05-02-85
